methods

Assay method for mitochondrial sterol 27-hydroxylase with 7α-hydroxy-4-cholesten-3-one as a substrate in the rat liver

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Abstract Mitochondrial sterol 27-hydroxylase (EC 1.14.13.15) is an important enzyme, not only in the formation of bile acids from cholesterol intermediates in the liver but also in the removal of cholesterol by side chain hydroxylation in extrahepatic tissues. The enzyme has been assayed by complicated methods using radiolabeled substrates or deuterium-labeled tracers. These methods may be inaccurate for measuring enzyme activity, because the amount of electron-transferring proteins may be insufficient for maximal velocity. To solve this problem, after solubilization of the enzyme from rat liver mitochondria with *n*-octyl-β-D-glucopyranoside (OGP), we measured the enzyme activity by incubating the solubilized enzyme with saturated amounts of electron-transferring proteins. In our assay system, using 7α-hydroxy-4-cholesten-3-one (HCO) as a substrate, we could easily measure the product, 7α,27-dihydroxy-4-cholesten-3-one, with HPLC monitoring absorbance at 240 nm. The product formation was proportionate to the time up to 5 min and the protein concentration up to 0.5 mg of protein/ml. The maximal velocity of the enzyme was 1.1 nmol/min/mg of protein, which was 4- to 16-fold higher than previously reported values. A simple and accurate assay method for sterol 27-hydroxylase in rat liver mitochondria is herein described.—Ota, Y., T-A. Eto, S-I. Tanaka, H. Sueta, H. Shiotsuki, Y. Maeda, M. Une, and K. Chijiiwa. **Assay method for mitochondrial sterol 27-hydroxylase with 7α-hydroxy-4-cholesten-3-one as a substrate in the rat liver. *J. Lipid Res.* 2003.** 44: **2400–2405.**

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It is well known that sterol 27-hydroxylase (CYP27A1) has an important role in the oxidation of the side chain of sterols in the formation of bile acids from cholesterol in the liver (1–4). In addition, the enzyme has another important role in the removal of cholesterol by side chain hydroxylation (5). Furthermore, this enzyme is present not only in the liver but also in extrahepatic tissues, i.e., brain (5), lung alveolar macrophages (6–9), endothelial cells (7, 10–12), ovary (13), intestine, skin, long bone, and calvaria (14).

To measure enzyme activity, 5β-cholestane-3α,7α,12α-triol (C-triol) has been used as a substrate. However, this assay system requires radiolabeled substrate (2–4) or deuterium-labeled tracers in the gas chromatography-mass spectrometry with selected ion monitoring (GC-SIM) methods (15–17). These assay methods are complicated and time-consuming. Another method using exogenous and endogenous cholesterol as a substrate was developed (18), which requires an oxidation step to quantify in HPLC. To measure the activity more simply and effectively, we used 7α-hydroxy-4-cholesten-3-one (HCO) as a substrate and were able to monitor its 27-hydroxylated product at 240 nm in HPLC.

In addition, when the mitochondrial suspension was used as an enzyme source, the maximal velocity could not be measured, because electron-transferring proteins were not saturated (19, 20). To solve this problem, we solubilized the enzyme from the rat liver mitochondria and fortified it with saturated amounts of adrenodoxin and NADPH-adrenodoxin reductase as the electron transfer system. In this study, we describe a simple and accurate assay method for mitochondrial sterol 27-hydroxylation.

MATERIALS AND METHODS

Chemicals

Adrenodoxin was prepared from bovine adrenals according to the method described by Suhara, Takemori, and Katagiri (21),

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Abbreviations: C-triol, 5β-cholestane-3α,7α,12α-triol; HCO, 7α-hydroxy-4-cholesten-3-one; GC-SIM, gas chromatography-mass spectrometry with selected ion monitoring; OGP, *n*-octyl-β-D-glucopyranoside; TMS, trimethylsilyl.

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and NADPH-adrenodoxin reductase was prepared by the modified methods of Suhara et al. (22), and Hiwatashi, Ichikawa, and Yamano (23). HCO was synthesized according to the method of Björkhem et al. (24). NADPH was from Oriental Co. (Tokyo, Japan), *n*-octyl-β-D-glucopyranoside (OGP) was from Sigma (St. Louis, MO), and pepstatin and leupeptin were from Wako Pure Chemical Industries (Osaka, Japan). All other reagents were of the highest grade commercially available. A high-performance liquid chromatographer was obtained from Shimazu (Kyoto, Japan, Model LC-10A) and equipped with a UV detector (Shimazu, SPD-10A) and an integration system (Shimazu, CLASS-LC10).

Animals

Animals used were two-month-old female rats, Sprague-Dawley strain, which were fed a laboratory rat chow. The animal protocol was approved by the Committee on Animal Experimentation of Miyazaki Medical College.

Synthesis of (25R,S)-7α,26-dihydroxy-4-cholesten-3-one

(25R,S)-5β-cholestane-3α,7α,26-triol (I) was synthesized as described previously (25). To a solution of I (200 mg) in benzene (15 ml) and acetone (9 ml), aluminium tri-*tert*-butoxide (240 mg) was added. The reaction mixture was refluxed for 3 h and poured into distilled water (200 ml). After acidification with diluted HCl, the reaction mixture was extracted with ethyl acetate (100 ml) and ethyl ether (100 ml). The combined organic layer was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness to give a residue (190 mg). The mixture was chromatographed on a silica gel column (50 g) using a solvent system of ethyl acetate graded into benzene. Elution with 40% ethyl acetate in benzene gave a fraction containing 7α,26-dihydroxy-5β-cholestan-3-one (II, 96 mg).

A mixture of II (90 mg), selenium dioxide (25 mg), and 95% ethanol (40 ml) was refluxed for 12 h. The reaction mixture was filtered, and the solvent was evaporated to dryness. The resulting residue was chromatographed on a silica gel column (30 g) using a solvent system of ethyl acetate graded into benzene. Elution with 90% ethyl acetate in benzene gave a fraction containing (25R,S)-7α,26-dihydroxy-4-cholesten-3-one (III, 32 mg); melting point: 191–192°C (acetone); NMR ($CDCl_3$) (δ-ppm): 0.72 (s, 3H, 18-CH_3), 0.91(d, 6.8Hz, 3H, 27-CH_3), 0.92 (d, 6.8Hz, 3H, 21-CH_3), 3.44 and 3.5 (dd, 2H, 26-CH_2OH), 3.97 (m, 1H, 7β-H), 5.81 (s, 1H, 4-H); GC-MS [trimethylsilyl (TMS) ether], *m/z* (intensity): 545 ($M^+ -15$, 49%), 470 ($M^+ -90$, base peak), 359 ($M^+ -$side chain, 21%), 269 ($M^+ -$side chain -90, 25%).

Preparation of mitochondrial fraction from rat liver

Rat liver was excised and homogenized with 9 vol of 15 mM Tris-HCl (pH 7.4) containing 0.19 M sucrose, 20% glycerol, 1 mM EDTA, 1 mM DTT, and 2 mg/ml each of leupeptin and pepstatin. The homogenate was centrifuged at 600 *g* for 15 min. The supernatant was then centrifuged at 8,000 *g* for 15 min. The pellets were washed once with the same buffer and then suspended in 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol, 1 mM EDTA, 1 mM DTT, and 2 mg/ml each of leupeptin and pepstatin to give a protein concentration of 15 mg/ml.

Solubilization of sterol 27-hydroxylase from rat liver mitochondria

Ten percent OGP dissolved in the mitochondrial suspension buffer was added dropwise to the mitochondrial suspension to give a final OGP:protein ratio of 4:5 (w/w). After being gently stirred for 30 min, the OGP-treated suspension was centrifuged at 100,000 *g* for 60 min, and the supernatant was used as an enzyme source. To test recovery of the enzyme activity in these procedures, the activity with the supernatant was compared with that of the OGP-treated mitochondrial suspension by the method described below; over 98% of enzyme activity was observed in the supernatant. Similar results were obtained by three independent experiments. All procedures were carried out at 4°C.

Enzyme assay

A typical incubation mixture contained an appropriate amount of enzyme, 5 nmol of adrenodoxin, 0.025 U of NADPH-adrenodoxin reductase, 20 μmol of potassium phosphate (pH 7.4), and 5 nmol HCO dissolved in 5 μl of ethanol, in a total volume of 0.25 ml. After the mixture was preincubated for 2 min at 42°C, incubation was initiated by adding 0.2 mmol NADPH and conducted for 5 min at the same temperature. The reaction was terminated by adding 4 ml ethyl acetate. The incubation mixture was re-extracted with 4 ml ethyl acetate, and the combined extract was washed twice with water. The organic phase was evaporated under a stream of nitrogen. The residue was dissolved in 20 μl isopropanol, and a 15 μl aliquot was subjected to HPLC analysis. For routine assay, straight-phase HPLC on a silica gel column (Wakosil 5SIL, 4.6 × 250 mm, Wako) was performed with a solvent system, hexane-isopropanol (4:1; v/v), at a flow rate of 1.0 ml/min, monitoring absorbance at 240 nm. The amount of the product was determined by comparing the peak area with those of known amounts of the chemically synthesized authentic sample (III).

Data processing. A Lineweaver-Burk plot of initial velocities was obtained by the weighted method of Wilkinson (26).

Protein determination. Proteins were determined by the method of Lowry et al. (27). Bovine serum albumin was used as a standard.

RESULTS

Assay for mitochondrial HCO 27-hydroxylation

As shown in **Fig. 1A**, an intense peak at 9.2 min was obtained by incubation of the solubilized mitochondrial enzyme fortified with adrenodoxin and NADPH-adrenodoxin reductase with HCO as a substrate. When the boiled enzyme was used, the product peak was not formed (Fig. 1B). When an authentic sample was injected under the same conditions, the main peak at 9 min and its shoulder peak at 9.2 min were indicative of (25S)-7α,26-dihydroxy-4-cholesten-3-one and (25R)-7α,26-dihydroxy-4-cholesten-3-one (7α,27-dihydroxy-4-cholesten-3-one), respectively (Fig. 1C). The retention time of the latter corresponded to that of the product. For further confirmation of the product, the fraction at 9.2 min in the straight-phase HPLC (Fig. 1A) was collected and evaporated under nitrogen gas. The residue was dissolved in methanol and subjected to reverse-phase HPLC analysis using a column (Finepak Sil C18, 4.6 × 150 mm, JASCO, Tokyo) with a solvent system [methanol-water (9:1; v/v)], at a flow rate of 1.0 ml/min. The peak of the product again corresponded to the peak of the authentic sample (data not shown).

Optimization of assay conditions for mitochondrial 27-hydroxylation

Effects of incubation temperature and time. To study the effect of temperature, incubations were performed for 5 min at 25°C, 30°C, 37°C, 42°C, 45°C, and 50°C. The higher enzyme activities were observed at 37°C, 42°C, and 45°C (data not shown). Incubations were performed for varying incu-

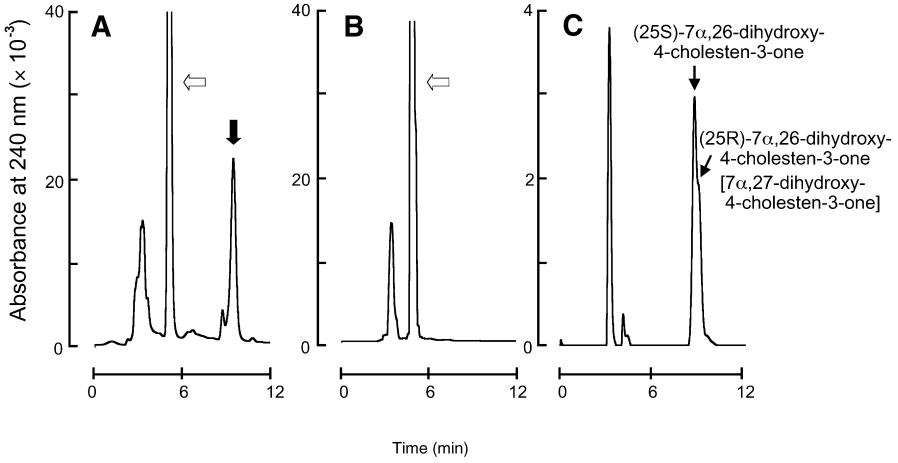

Fig. 1. Typical HPLC chromatograms of the products obtained by incubation of 7α-hydroxy-4-cholesten-3-one (HCO) with 100,000 *g* supernatant of the *n*-octyl-β-D-glucopyranoside (OGP)-treated liver mitochondria of the rat (A), and with boiled supernatant of the OGP-treated liver mitochondria as an enzyme source (B). Open arrow indicates HCO peak; solid arrow indicates the product. C: The HPLC chromatogram of the authentic (25R,S)-7α,26-dihydroxy-4-cholesten-3-one, 100 pmol; main peak indicates (25S)-7α,26-dihydroxy-4-cholesten-3-one, shoulder peak (25R)-7α,26-dihydroxy-4-cholesten-3-one (small arrows). In this system, 27-hydroxylated HCO was measurable down to 1 pmol.

bation times at these three temperatures (**Fig. 2**). When incubated at 42°C, the amount of 7α,27-dihydroxy-4-cholesten-3-one produced was always higher than that produced at the other temperatures, and increased linearly up to 5 min. Therefore, these conditions were selected for the standard assay method.

Effect of concentrations of the electron-transferring proteins. To study the effect of the concentrations of adrenodoxin and NADPH-adrenodoxin reductase on 27-hydroxylation of HCO, incubations were performed at varying concentrations of these proteins (**Fig. 3**). When reductase concentrations were ≥0.8 U/ml, the reaction velocity increased linearly, dependent on the concentration of adrenodoxin, and then leveled off. When reductase concentrations were ≤0.4 U/ml, the reaction velocity increased linearly with the concentration of adrenodoxin up to 5 μM, decreased at 10 μM, and then disappeared at ≥20 μM. Accordingly, 20 μM of adrenodoxin and 0.1 U/ml of NADPH-adrenodoxin reductase were adopted for routine assay.

Effect of enzyme concentration. To study the effect of enzyme concentration on 27-hydroxylation of HCO, incubations were performed at varying concentrations of the enzyme (**Fig. 4**). The rate of product formation was proportionate to the concentration of the enzyme up to 0.5 mg protein/ml. Above this concentration, there was no further linearity in the calibration curve. Therefore, 75–125 μg of protein/0.25 ml was used for routine assay.

Effect of substrate concentration. To study the effect of substrate concentration on the rate of 27-hydroxylation, incubations were performed at varying concentrations of HCO (**Fig. 5**). The enzyme reaction followed Michaelis-Menten kinetics. The maximal velocity was 1.1 nmol/min/mg of protein. The K_m value calculated from a Lineweaver-Burk plot was 4.8 μM. The incubation with 25 μM HCO was optimum for routine assay.

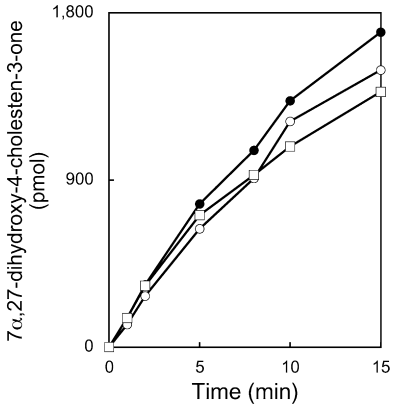

Fig. 2. Effect of incubation time on the production of 7α,27-dihydroxy-4-cholesten-3-one. Enzyme activity was measured using 125 μg protein. Incubation was conducted as described in Materials and Methods and incubation time was varied at different temperatures: 37°C (open circles), 42°C (closed circles), and 45°C (squares). Values were means of duplicate experiments.

DISCUSSION

In this paper, we establish a simple and accurate assay method for mitochondrial sterol 27-hydroxylation by using HCO as a substrate and by incubating the solubilized enzyme with saturated amounts of electron-transferring proteins. With this method, the maximal velocity of 1.1 nmol/min/mg of protein was obtained, which was 4- to 16-fold higher than those of previous reports (4, 17, 18, 28–30). To check the reproducibility of our method, we examined the activities in preparations of four different samples of the liver, with a resulting specific activity of 1,350 ± 30 pmol/min/mg of protein (mean ± SD). As shown in Fig. 4, our method showed good linearity at con-

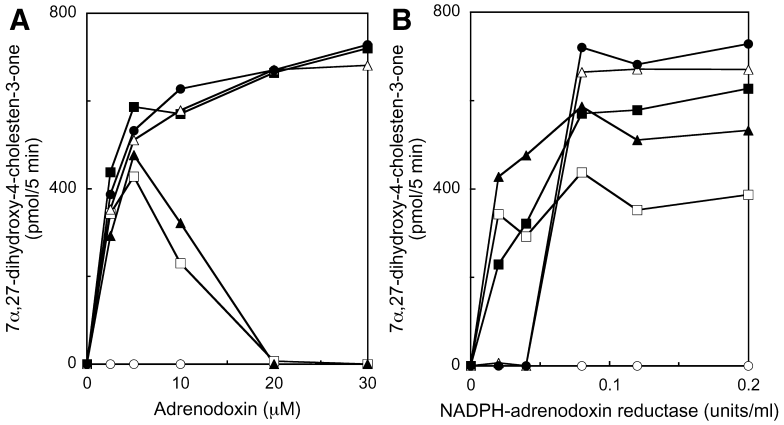

Fig. 3. Effect of concentrations of adrenodoxin (A) and NADPH-adrenodoxin reductase (B) on 27-hydroxylation of HCO. Enzyme activity was measured using 100 μg protein. The amounts of NADPH-adrenodoxin reductase used in experiment A were 0 U (open circles), 0.02 U (open squares), 0.04 U (closed triangles), 0.08 U (closed squares), 0.12 U (open triangles), and 0.2 U (closed circles). Amounts of adrenodoxin used in experiment B were 0 μM (open circles), 2.5 μM (open squares), 5 μM (closed triangles), 10 μM (closed squares), 20 μM (open triangles), and 30 μM (closed circles). Values were means of duplicate experiments.

centrations of up to 0.5 mg of protein/ml. These results indicate that this method has high reproducibility for assaying 27-hydroxylation of HCO. For confirmation of the incubation product obtained from our assay method, liquid chromatography-mass spectrometry was performed after the reverse-phased HPLC analysis. The peak of the incubation product was observed at m/e 417 (M^+) (data not shown), giving the molecular weight of this product as 416, which corresponds to that of 7α,27-dihydroxy-4-cholesten-3-one.

The most important advantage of our method is the use of HCO as a substrate for 27-hydroxylation. Use of HCO made possible the simple measurement of the reaction product by HPLC at an absorbance of 240 nm, which was the reason that we selected HCO from among other substrates. Okuda and Hoshita (2) developed the first assay method for mitochondrial 27-hydroxylation by using tritium-labeled C-triol as a substrate, which required separation of the products by TLC. Oftebro et al. (15) developed the GC-SIM method for assaying C-triol 27-hydroxylation using deuterium-labeled 5β-cholestane-3α,7α,12α,27-tetrol as an internal standard. To separate 5β-cholestane-3α,7α,12α,27-tetrol effectively, Honda et al. (17) developed the high-resolution GC-SIM by treating the incubation mixture with a silica cartridge column before conversion to TMS derivatives. Another assay method for mitochondrial 27-hydroxylation was developed using cholesterol as a substrate (18). This method required an additional oxidation step with cholesterol oxidase for measurement of the product at 240 nm in HPLC, and the specific activity was not as high as that achieved by our method, despite the use of 2-hydroxypropyl-β-cyclodextrin. These methods were more complicated and time-consuming than our method. In addition, Wikvall (28), using purified mitochondrial enzyme from rabbit liver mitochondria, showed results in which the 27-hydroxylation activity toward HCO was higher than that toward other steroids, such as cholesterol, indicating that HCO is preferable to cholesterol as a substrate.

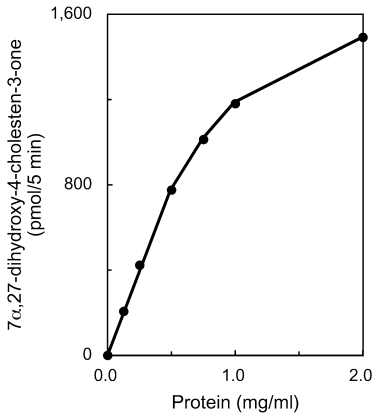

Fig. 4. Effect of enzyme concentration on the production of 7α,27-dihydroxy-4-cholesten-3-one. Incubation was carried out as described in Materials and Methods, except that the amount of enzyme was varied. Values were means of duplicate experiments. To test the reproducibility of the assay system, the enzyme activities were measured at protein concentrations of 0, 0.125, 0.25, and 0.5 mg/ml by triplication. The linearity was checked by simple linear regression, and the line satisfied the relationship y = 1,360x − 10 (n = 4, $r = 0.99$, $P < 0.002$).

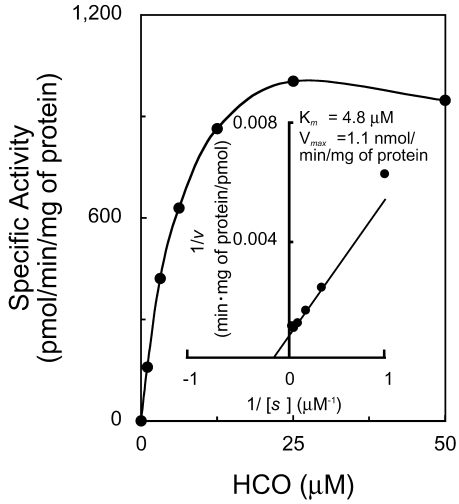

Fig. 5. Effect of substrate concentration on 27-hydroxylation of HCO. Enzyme activity was measured using 125 μg protein. Incubation was carried out as described in Materials and Methods, except that the concentration of substrate was varied. The inset shows the Lineweaver-Burk plot. The points are experimental, and the line is a weighted least-squares fit of the data to hyperbola. Values were means of duplicate experiments.

We hypothesized that HCO would be a suitable substrate for sterol 27-hydroxylase, because the serum levels of HCO are extremely high in patients with cerebrotendinous xanthomatosis (CTX) (31), a disease resulting from mutations in *CYP27A1*, the sterol 27-hydroxylase gene (32–36). In our study, high catalytic 27-hydroxylation activity for HCO was obtained. Therefore, it was interesting to speculate that the increased serum levels of HCO in patients with CTX were not only due to increased 7α-hydroxylation of cholesterol but also to decreased 27-hydroxylation of HCO.

To assay mitochondrial P450 accurately, saturation of electron-transferring proteins is necessary (19, 20). When inadequate quantities of adrenodoxin and NADPH-adrenodoxin reductase were used, 27-hydroxylase activity could not be measured accurately (Fig. 3). This indicates that the enzyme activity might be underestimated if mitochondrial suspension is used as an enzyme source. When a lower concentration of the reductase was used under excessive concentrations of adrenodoxin, much lower reaction velocity than expected was obtained (Fig. 3). It could be assumed that under such conditions, the iron-sulfur protein could not accept electrons effectively.

To fortify the enzyme with electron-transferring proteins, it is necessary to solubilize the enzyme from mitochondrial inner-membrane matrix (37), because exogenous electron-transferring proteins might not reach the enzyme. Furthermore, with intact mitochondria, the rate of transport of the substrate to the mitochodrial inner membrane might affect the catalytic activity. Solubilization of the enzyme from the mitochondria can eliminate this problem.

We found that OGP was the most suitable detergent, because others, such as cholic acid, CHAPS, polyethylene glycol monododecyl ether (Lubrol PX), and polyoxyethylenesorbitan monolaurate (Tween 20), had inhibitory effects on 27-hydroxylation of HCO (data not shown).

In summary, we have developed a simple and accurate assay method for mitochondrial sterol 27-hydroxylation. This method would be helpful in investigating the regulation and physiological role of this enzyme in the liver and extrahepatic tissues.

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